ENERGY DEPENDENCE OF PYRIDINE NUCLEOTIDE-LINKED DISMUTATIONS IN RAT LIVER MITOCHONDRIA

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In previous studies (Azzone and Ernster, 1961) it was shown that the aerobic oxidation of α-ketoglutarate (αKG) in rat liver mitochondria in the presence of 2,4-dinitrophenol requires P_i but not ADP for maximal activity. Arsenate, which abolishes the αKG-linked substrate-level phosphorylation, was able to replace P_i in inducing maximal αKG-oxidation. The lack of requirement for ADP in addition to P_i was interpreted as being due to a continuous regeneration of mitochondrially bound ADP from ATP by way of the dinitrophenol-induced ATPase. A possible role of succinate oxidation in mediating interaction between intramitochondrial ATP and the dinitrophenol-induced ATPase was suggested.

The present paper describes experiments in which the role of P_i and phosphate acceptor in the anaerobic dismutation of αKG + NH_3 to succinate + CO_2 + glutamate was investigated. This dismutation was first described by Krebs and Cohen (1939) and was later used by Hunter and Hixon (1949) to demonstrate the phosphorylation linked to the substrate-level oxidation of αKG. In this system, succinate is not oxidized further, and addition of dinitrophenol, which was needed in the aerobic system to eliminate the P_i- and ADP-requirement of respiratory chain phosphorylations, is not necessary. As will be shown below, in this anaerobic dismutation also, only P_i, and not ADP, is required for maximal rate of αKG-oxidation. The evidence to be presented supports the conclusion that a high-energy phosphate intermediate formed in the αKG-linked phosphorylation is utilized for the transfer of hydrogen from the αKG dehydrogenase to the glutamate dehydrogenase system, or in general to other dehydrogenase systems.

Experimental.- Rat liver mitochondria were prepared according to Ernster and Löw (1955). Incubations were made in open test-tubes, where anaerobiosis was created by the addition of antimycin A. The standard system also contained Rotenone, in order to prevent succinate-linked pyridine nucleotide reduction (cf. Ernster et al., 1962). After terminated incubation the samples were fixed with 0.2 ml 25 % perchloric acid and αKG was determined according to Friedemann and Haugen (1943). Esterification of P_i was estimated by the isotope distribution method described by Lindberg and Ernster (1955). $P/2e^-$ is expressed as the ratio of μmoles P_i esterified to μmoles αKG oxidized (i.e., half the amount of αKG removed in the dismutation involving αKG + NH_3, and the total amount of αKG removed in the dismutation involving αKG + acetoacetate).

Table I.

Effect of P_i on the Anaerobic Dismutation of αKG + NH_3 in Rat Liver Mitochondria.

Each tube contained 7.6 mg mitochondrial protein, 6 mM αKG, 3 mM NH_4Cl, 40 mM KCl, 20 mM tris buffer (pH 7.5), 2 mM $MgCl_2$, 50 mM sucrose, 2 μg antimycin A, and 0.32 μg Rotenone, in a final volume of 2 ml. Incubation at 30°C for 20 min.

P_i added, mM	$-\Delta$ αKG, μmoles
0	1.60
0.02	2.32
0.1	2.80
0.5	4.08
2	4.92
10	3.04

Results.- As shown in Table I, added P_i stimulated the anaerobic rate of αKG removal in the presence of NH_3 about 3-fold. Maximal enhancement occurred at a P_i concentration of 2 mM; 10 mM P_i was less efficient. It may be noted that as little as 0.02 mM (0.04 μmoles per sample) P_i gave an increase in αKG-removal of 0.7 μmoles, indicating that there was no stoichiometric relationship between the added amount of P_i and the enhancement of αKG-removal.

Fig. 1a shows that the αKG-removal was largely linear with time, both in the absence and presence of P_i. Addition to the P_i-supplemented system of ADP

or ADP + AMP (both in the presence of glucose and hexokinase) did not alter the rate of αKG-removal. Fluoride (40 mM) abolished the enhancement of the αKG-removal by P_i, both in the absence (not shown) and in the presence of ADP, glucose and hexokinase.

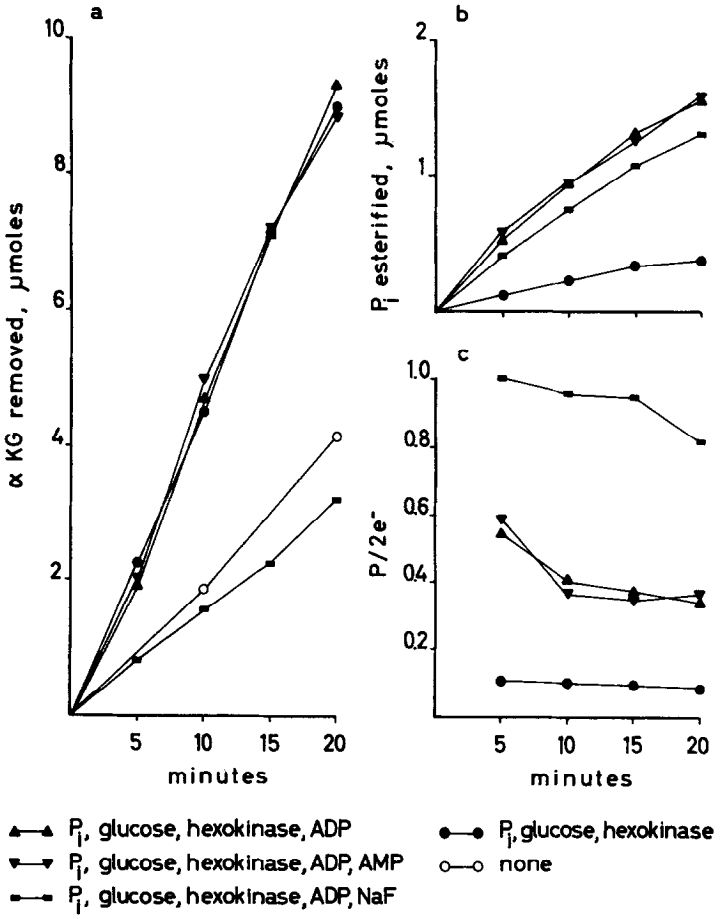

Fig. 1 Effects of P_i. Phosphate Acceptor and Fluoride on αKG Removal and Coupled Phosphorylation During the Anaerobic Dismutation of αKG + NH_3 in Rat Liver Mitochondria.

Each tube contained 8 mg mitochondrial protein, 5 mM αKG, 5 mM NH_4Cl, 40 mM KCl, 20 mM tris buffer (pH 7.5), 2.5 mM $MgCl_2$, 50 mM sucrose, 2 μg antimycin A, 0.32 μg Rotenone, and, where indicated, 2 mM P_i^{32}, 1 mM ADP or 0.5 mM ADP + 0.5 mM AMP, 30 mM glucose, 150 KM units of yeast hexokinase, and 40 mM NaF, in a final volume of 2 ml. Incubation at 30°C.

In the presence of ADP, glucose and hexokinase, there occurred a net esterification of P_i (Fig. 1b). This was linear or slightly declining with

time and was virtually unaffected by fluoride. Since fluoride strongly depressed the rate of αKG-removal, this implied a substantial increase in $P/2e^-$ ratio. Indeed, as shown in Fig. 1c, this ratio was in the vicinity of 1 (the theoretical maximum for the substrate-level oxidation of αKG) in the presence of fluoride, whereas it was only 0.3-0.5 in its absence. The latter low ratio could not be increased if AMP was used in addition to (or instead of) ADP (cf. Azzone and Ernster, 1961).

Oligomycin and dinitrophenol, at levels which are known to abolish respiratory chain phosphorylation, did not inhibit the phosphate uptake in the present system, either in the presence or absence of fluoride (Table II). This finding is in accordance with earlier observations concerning the insensitivity of the substrate-level phosphorylation to dinitrophenol (Hunter, 1951) and oligomycin (Chappell and Greville, 1961). However, both dinitrophenol and oligomycin did depress the rate of αKG-removal, thus causing an increase in the $P/2e^-$ ratio. These effects were not additive to that of fluoride. When arsenate was added to the P_i-ADP-glucose-hexokinase-supplemented system it abolished, as it should, the P_i uptake, and simultaneously lowered the rate of αKG-removal to the fluoride-inhibited level; the latter was unaffected.

Table II.

Effects of Oligomycin, 2,4-Dinitrophenol and Arsenate on the Anaerobic Dismutation of αKG + NH_3 in the Absence and Presence of Fluoride.

Each vessel contained 10 (Expt. 1) or 8 (Expt. 2) mg mitochondrial protein, 2 mM P_i^{32}, 1 mM ADP, 30 mM glucose, and 150 KM units of yeast hexokinase. Other conditions were as in Fig. 1. Time of incubation was 10 min. in Expt. 1 and 20 min. in Expt. 2.

Expt. No.	Additions	Without fluoride			With 40 mM fluoride		
		$-\Delta$ αKg, µmoles	$-\Delta P_i$, µmoles	$P/2e^-$	$-\Delta$ αKg µmoles	$-\Delta P_i$, µmoles	$P/2e^-$
1	None	7.28	1.15	0.32	2.35	0.84	0.72
	Oligomycin, 2 µg/sample	4.56	1.42	0.62	2.06	0.76	0.74
	" , 4 " / "	3.77	1.46	0.78	2.40	0.84	0.70
	Dinitrophenol, 0.01 mM	4.68	1.08	0.46	2.00	0.72	0.72
	" , 0.1 "	2.55	0.94	0.74	1.80	0.80	0.89
2	None	9.81	1.27	0.26	4.69	1.85	0.79
	Arsenate, 10 mM	5.50	0.12	0.04	4.05	0.15	0.07

In an experiment reported in Table III, NH_3 was replaced by acetoacetate, thus using the β-hydrobutyric dehydrogenase rather than the glutamic dehydrogenase reaction as a trap for the reducing equivalents derived from αKG. Also in this system, the rate of αKG-removal was markedly depressed by 40 mM fluoride, with a simultaneous increase in the $P/2e^-$ ratio.

Table III.

Effect of Fluoride on the Anaerobic Dismutation of αKg + NH_3 and of αKG + Acetoacetate in Rat Liver Mitochondria.

The composition of System I was as in Table II, Expt. 2. System II contained the same additions as System I, except that NH_4Cl was omitted and 5 mM acetoacetate was added. Time measured, 20 min.

System	fluoride	$-\Delta$ αKG μmoles	$P/2e^-$
I. 2 αKG + NH_3 ⟷	-	9.75	0.31
succ. + CO_2 + glut.	+	3.89	0.79
II. αKG + AcAc ⟷	-	4.42	0.46
succ. + CO_2 + βOH	+	1.44	1.02

Discussion.- When Hunter and Hixon (1949) first demonstrated the α-ketoglutarate-linked substrate-level phosphorylation they used 40 mM fluoride in their system. Our results show that this is a necessary addition in order to obtain a satisfactory $P/2e^-$ ratio. Simultaneously with the increase in $P/2e^-$ ratio, fluoride caused a marked decrease in the rate of αKG-removal. Similar effects were obtained with oligomycin and dinitrophenol, and these were not additive to the effect of fluoride. Furthermore, arsenate, which uncouples the substrate-level phosphorylation, depressed the rate of αKG-removal from the maximal to the fluoride-inhibited level. Our tentative interpretation of these results is that a high energy phosphate compound formed in the αKG-linked substrate-level phosphorylation serves as a source of energy for driving pyridine nucleotide-mediated dismutations in rat liver mitochondria. The energy utilizing process, which is blocked by fluoride, appears to constitute a powerful means of splitting

this high energy phosphate compound since addition of phosphate acceptor did not affect the maximal dismutation rate. Significantly, the route of energy-transfer seems to involve the oligomycin- and dinitrophenol-sensitive reactions of the mitochondrial energy-transfer system. Tager (1962) has recently reported evidence indicating a requirement of high-energy compounds generated by the respiratory chain for a maximal rate of dismutation between malate and αKG + NH_3. The present results are analogous to those of Tager except that here a high-energy phosphate intermediate, generated by the substrate-level phosphorylation, supplies the energy. Similarly to Tager, we are inclined to interpret our results in terms of a requirement for high-energy bonds in transferring hydrogen from one mitochondrial pyridine nucleotide compartment to another. In a succeeding communication (Danielson and Ernster, 1962) we shall present direct evidence for an energy-dependent pyridine nucleotide transhydrogenase reaction in mitochondria.

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